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SIMULTANEOUS FERMENTATION AND ISOMERIZATION OF XYLOSE TO ETHANOL

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ABSTRACT

Xylose was fermented anaerobically to ethanol by combining the enzyme, xylose isomerase, with the yeast Schizosaccharomyces pombe in pH, and temperature controlled fermenters. The effects of media composition, temperature, pH and enzyme loading were examined. Significant improvement (15 to 30%) in ethanol yields were found when the fermentations were run at lower temperatures (28°C.) and at the lowest pH that allowed for reasonable enzyme activity (pH 5.5). Since fermentation at low pH is necessary for high yields, recombinant E. coli cells containing overproduced xylose isomerase were immobilized in a chitosan matrix to provide for high activity at low pH; the immobilized cells were found to have four times the specific activity at pH 5.5 than non-immobilized cells, and an estimated 40 times the activity of non-recombinant, xylose induced cells.

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INTRODUCTION

Fermentation of the xylose found in the hemicellulose fraction of agricultural and hard wood biomass is a key economic factor in the production of ethanol from these feed stocks. Xylose, the major component in hemicellulose, can amount to as much as one third of the fermentable sugar that is obtained from these raw materials. Xylose is relatively easy to obtain by dilute acid pretreatment of the biomass wastes but the sugar is more difficult to ferment to ethanol than the glucose derived from the cellulose fraction. Few yeasts or fungi, and bacteria can ferment xylose but, in general, low yields and/or rates of ethanol production are found.

The most promising microorganisms for xylose conversion to ethanol are yeasts. Recently, several yeast strains, i.e. <u>Pachysolen</u> <u>tannophilus</u>, <u>Candida</u> <u>shehatae</u> and <u>Pichia</u> <u>stipitis</u>, (duPreez 1986, Chung 1986, Beck 1986). were found to produce reasonable yields of ethanol from xylose or wood hydrolyzates when the fermentations were run in the presence of small amounts of oxygen. In yeast, xylose is initially converted to the alcohol sugar, xylitol, and then to the ketose sugar, xylulose. The addition of oxygen is apparently required for the oxidation of the NADH produced in the conversion of xylitol to xylulose which is mediated by the NAD specific enzyme, xylitol dehydrogenase (Bruinenberg 1983).

Without oxygen, xylitol is produced as a major byproduct; with too much oxygen, the yeasts convert the sugar to CO_2 and H_2O instead of to ethanol and also utilize ethanol made previously by fermentation for growth under aerobic conditions. Thus, precise control of oxygen level in these fermentations is required. Also, these strains are not as ethanol tolerant as those that are traditionally used in fermentation of sugars to ethanol, i.e. <u>Saccharomyces</u> <u>cerevisiae</u> or <u>Schizosaccharomyces</u> <u>pombe</u>. Yields in these fermentations begin to drop dramatically when the substrate concentration exceeds approximately 40 g/L.

It would be preferable to perform xylose fermentation without the addition of oxygen. Totally anaerobic fermentation of xylose to ethanol can be achieved by enzymatically converting the xylose to a more fermentable form, xylulose, using the enzyme xylose isomerase (Chiang 1981a,b, Gong 1981a,b, Hahn-Hagerdal 1986, Hsiao 1981, Jeffries 1981, Maleszka 1982, Roman 1984, Schneider 1981, Wang 1980a,b). However, since the reaction is reversible, and at equilibrium only about 15 to 20% of the mixture is xylulose, it is important to continuously remove the xylulose from the reaction to complete the isomerization. This is readily done by adding the enzyme to yeast fermentations of xylose. The simultaneous fermentation and isomerization of xylose, or SFIX, allows for the removal of xylulose by the yeast as they ferment the sugar to ethanol.

When yeasts ferment xylulose, the activity of xylitol dehydrogenase in no longer required, and the need for oxidation of NADH is eliminated. Yeasts that are ethanol tolerant can be used in these anaerobic fermentations, and, in addition, the theoretical yield of ethanol from xylulose is 10% higher than when xylose is fermented in the presence of oxygen. Thus, potentially higher ethanol yields can be produced anaerobically. Fermentation technology and genetic engineering are being combined at SERI to achieve these higher yields.

EXPERIMENTAL DESCRIPTION

Fermentations were performed in a bank of six New Brunswick Multigen Model F-1000 fermenters, that provided for agitation and temperature control, at working volumes of 500 mL. Each fermenter was equipped for pH control: Ingold 465-K9 electrodes, Cole-Palmer J-7537 pumps with adjustable time delay and Cole-Palmer Type 5997-30 pH controllers.

Sweetzyme Q (xylose isomerase) was a gift from NOVO Laboratories. Overproduction of xylose isomerase in recombinant _E. coli_ has been previously described (Lastick 1986); the cells containing the overproduced enzyme were lyophilized and stored at -20°C for use in SFIX experiments or for cell immobilization. Ethanol concentration was measured by gas chromatography (Hewlett Packard 5880A, Porapaq Q 80/100 column) using isopropanol as an internal standard. Sugars were measured by ion-mediated HPLC (Biorad HPX-87C) using mannitol as an internal standard. Fermentation data were analyzed on a Lotus 1-2-3 spreadsheet. Xylose isomerase activity was measured by incubation at 37°C for 1 hr. with 15mM xylose, 50 mM MOPSO (pH 7.5), 10 mM $MgSO_4$, 1mM $CoCl_2$; xylulose produced was assayed using the "cysteine carbazole" procedure (Horecker 1974). Lyophilized, recombinant LE392/pRK248/pTXI-1 _E. coli_ cells (Lastick 1986) were immobilized by entrapment in a chitosan matrix (Vorlop 1981).

Yeast strains were obtained from the U.S. Department of Agriculture, Northern Regional Research Laboratories, Peoria, Illinois or from the American Type Culture Collection, Rockville, Maryland. Seed cultures for SFIX experiments were grown at 32°C to the late log stage of growth on 1% yeast extract, 2% bactopeptone and 2% dextrose (YEPD). The cells were then centrifuged (8000g/10 min.) and resuspended in media containing xylose (usually 6%), yeast extract and bactopeptone as before (YEPX), Sweetzyme Q or lyophilized, recombinant _E. coli_ cells containing overproduced xylose isomerase. Tetracycline (15 mg/L) and ampicillin (60 mg/L) were added to discourage bacterial growth; $MgSO^4$ and $CoCl_2$ were also added to the medium, at final concentrations of 10mM and 1mM respectively, to support enzymatic activity.

RESULTS

Screening yeast strains for SFIX

Seven yeast strains were initially screened for fermentation of 6% xylose to ethanol, at 30°C with 10 g/L NOVO Sweetzyme Q: 2 strains of _Schizosaccharomyces pombe_, Y-164, ATCC-26192, 2 strains of _Saccharomyces cerevisiae_, ATCC-24860, SERI-D5A, 2 strains of _Candida tropicalis_, Y-11860, Y-1552, and _Pichia stipitis_ ATCC-58784 (Fig. 1). _S. pombe_ Y-164 produced ethanol at the fastest rate, and at the highest yield. The fermentation of Y-164 was repeated and the rate and yield were reproduced.

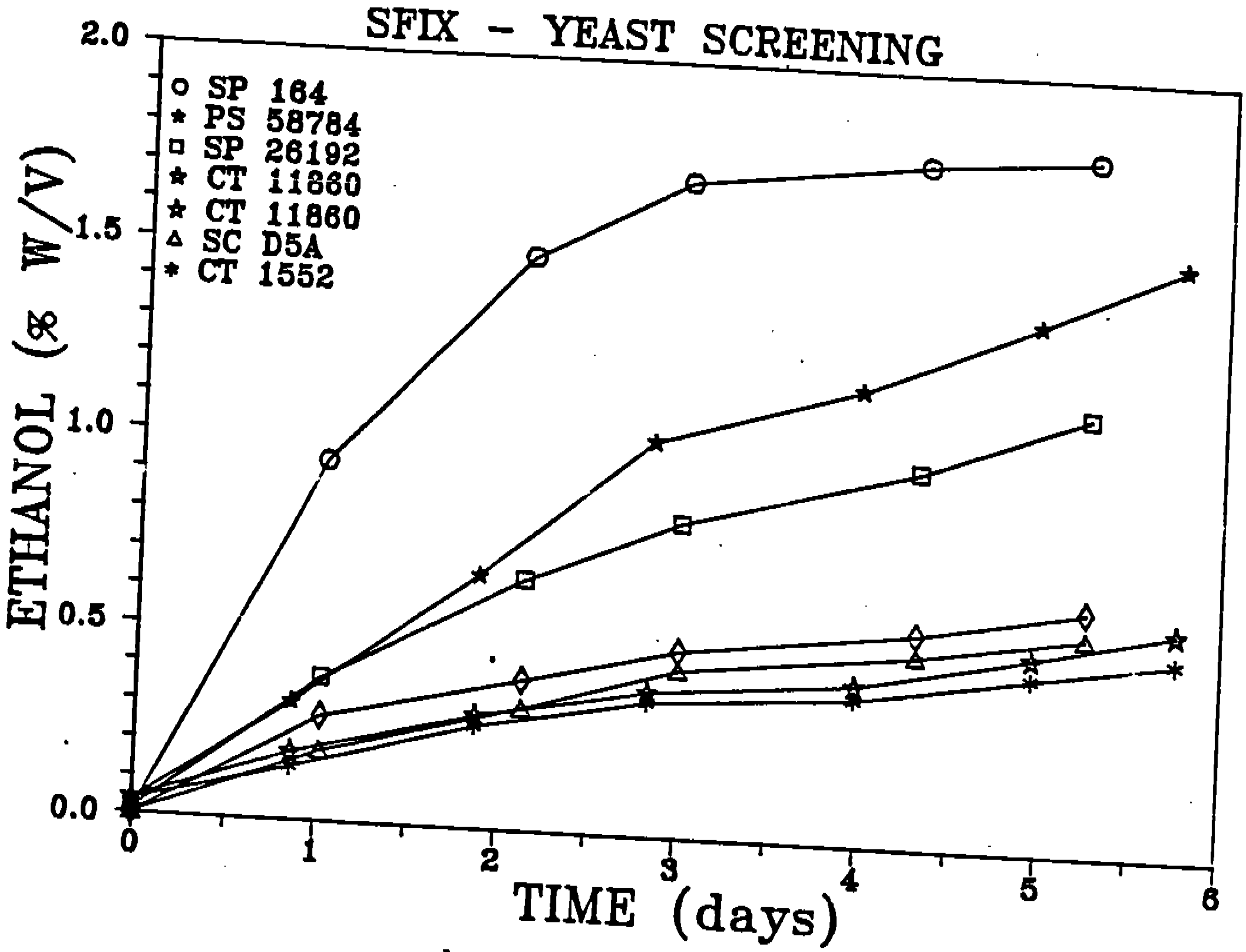

Figure 1. Cells grown to stationary phase in YEPD medium were used to ferment 6% xylose (YEPX) at 30°C., pH-6.1, with 10 g/L Sweetzyme Q. See results for sources of the yeast strains.

Since *S. pombe* Y-164 seemed to be well suited for SFIX optimization and, in addition, is an extremely flocculent strain, which would allow for cell recycle, it was chosen for further study.

Effect of temperature on SFIX

S. pombe cells were found to grow efficiently on YEPD medium at temperatures as high as 37°C. However, an inverse relationship was found between fermentation temperature and ethanol yield when xylose is fermented by SFIX. A series of fermentations were run at the same time, using exactly the same medium (YEPX, 6% xylose), pH (6.1), enzyme loading (0.4 g/L = 200 IU/L, *E. coli* JC1553/pRK248-pTXI-1), and inoculum (1.5 g/L dry weight), at temperatures of 28°, 30°, 32°, and 35° C (Fig. 2). Reduced yields were the result of increased loss of yeast viability at higher temperatures. The loss in viability was manifested by reduction in xylose utilization. Microscopic examination of the culture using phase contrast optics showed increased cell lysis, reduction in the number of dividing cells, and the increased presence of inclusion vacuoles and phase dense granules.

Enzyme loading in SFIX

Sweetzyme Q. Previous experiments on a number of commercial glucose isomerases indicated that Sweetzyme Q, an immobilized whole cell preparation of *Bacillus coagulans* from NOVO Laboratories, was more active in xylose isomerization than the other glucose isomerases tested and has been used previously for SFIX fermentation (Chaing 1981a,b, Gong 1981a,b, Hsiao 1982, Jeffries 1981, Roman 1984). Controlled fermentations (see conditions above) were run using 2, 5, 7, 10, 15 and 20 g/L dry weight Sweetzyme Q at 28°C. Ethanol production and yield was found to increase as enzyme loadings from 2 to 10 g/L, with small improvements seen at higher loadings (Fig. 3).

A similar experiment was performed at a pH of 5.5 instead of pH 6.1 (not shown). Recent experiments (see below, Effect of pH on SFIX), and a report in the literature (Roman 1985) indicate that this immobilized enzyme preparation maintains, at pH 5.5, a significant level (40%) of its activity at the optimal pH (7.5). Optimal enzyme loading at pH 5.5 was at 20 g/L, as might be expected due to the lower specific activity. This observation is important with respect to the effect of pH on the SFIX process, as described below.

E. coli JC1553/pRK248-pTXI-1. The lyophilized cells, assayed at a specific activity of 0.5 I.U./mg protein (37°C., pH 7.5), were used at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 g/L; the sixth fermentation was loaded with 10 g/L sweetzyme Q for comparison. The fermentations were run as before at 28°C., pH 6.1. It was found that loading of the lyophilized *E. coli* cells produced a maximum ethanol yield at 0.8 g/L (400 IU/L) but, unlike the immobilized sweetzyme Q, at higher concentrations of *E. coli* cells the fermentations produced significantly less ethanol (Fig. 4).

In these lyophilized preparations there is a real possibility that, since many of the *E. coli* cells remain viable after induction for enzyme overexpression and lyophilization, the cells can secrete factors that inhibit yeast viability. Assimilation of the xylose by the viable *E. coli* is unlikely in this case since this strain is deficient in xylulokinase, an enzyme

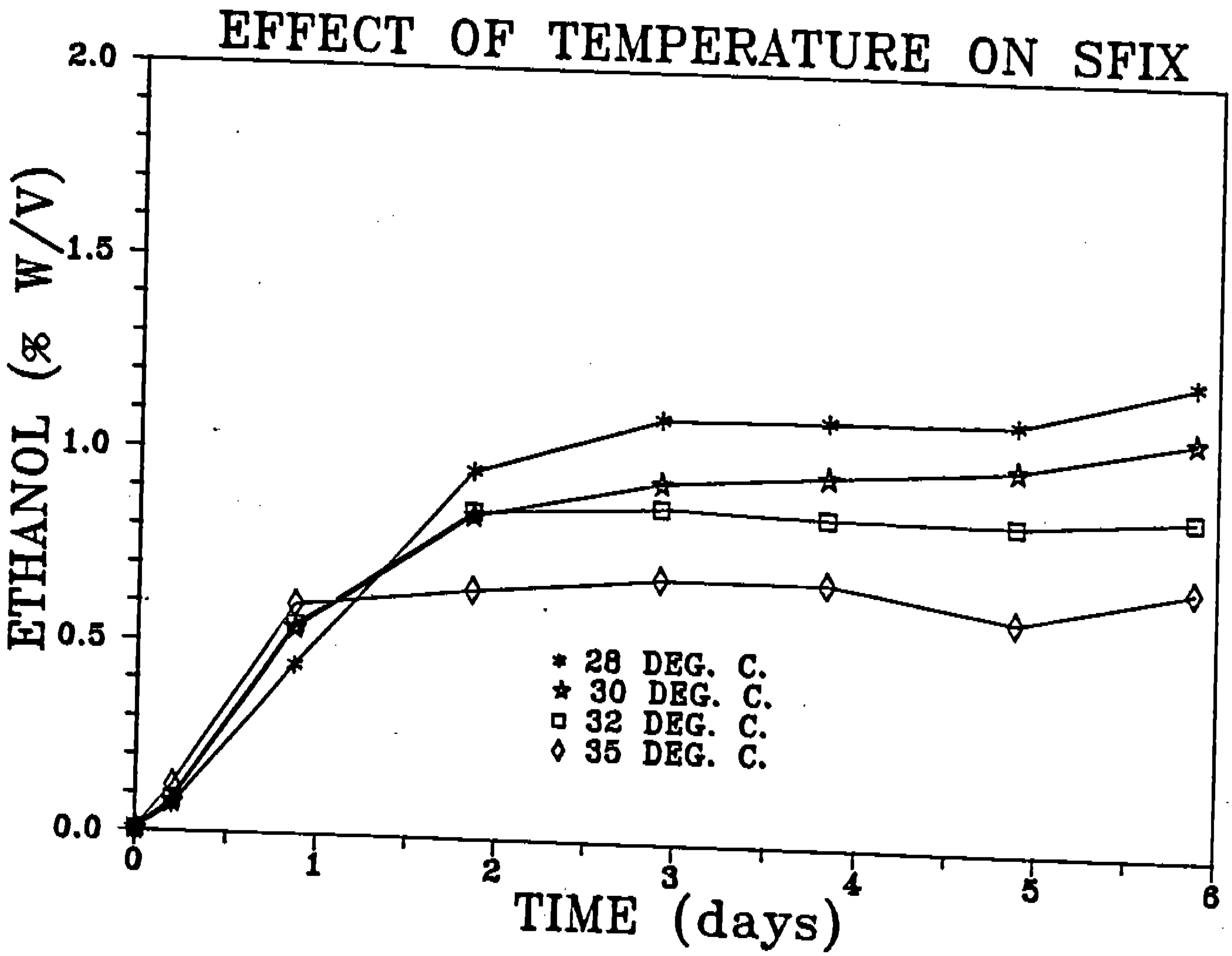

Figure 2. S. pombe Y164 was used to ferment 6% xylose (YEPX) at pH 6.1 using 0.4 g/L (200 IU/L) E. coli JC1553/pTXI-1-pRK248 lyophilized cells, at 28°, 30°, 32°, and 35° C.

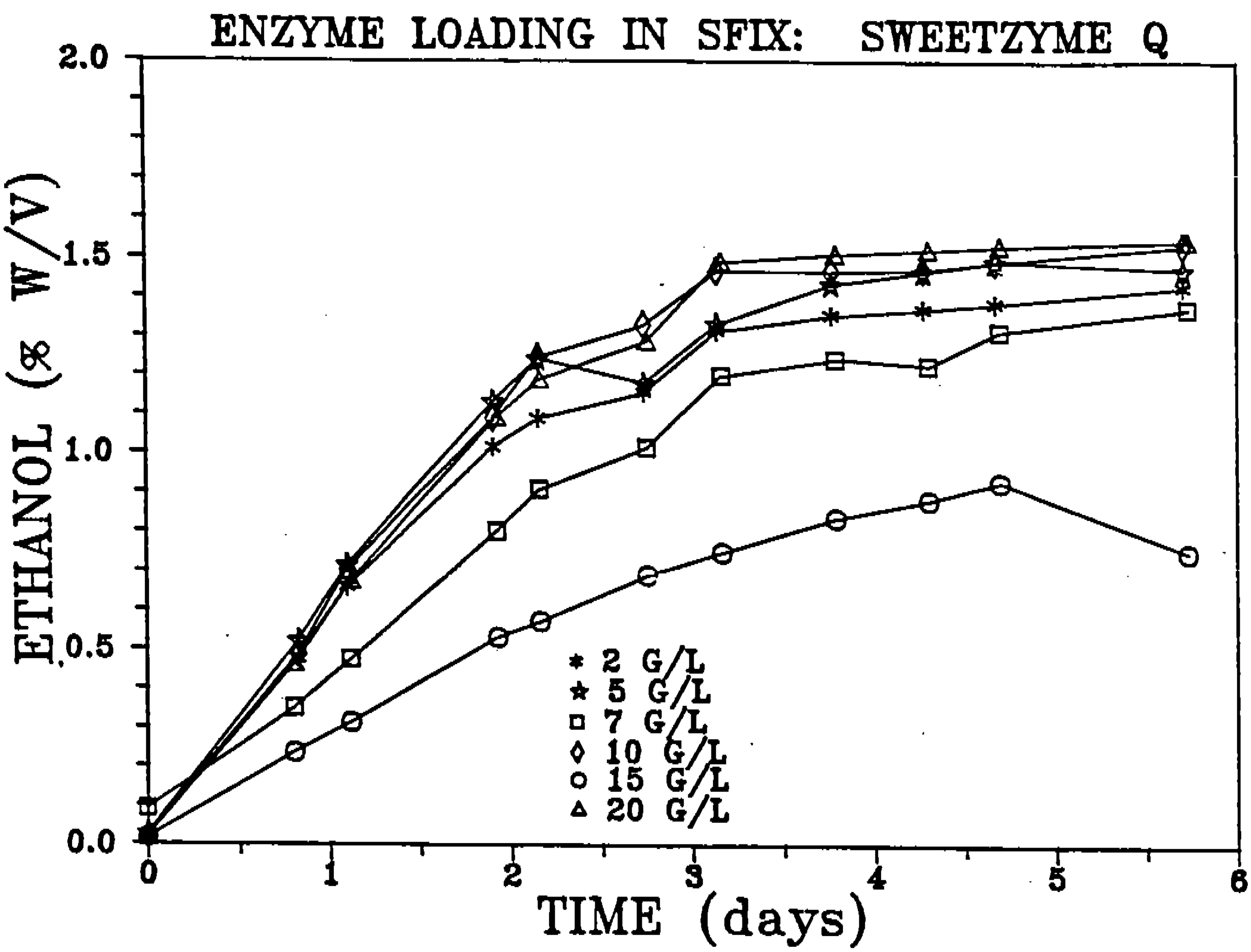

Figure 3. S. pombe Y164 cells were used to ferment 6% xylose (YEPX) at 28°C., pH6.1, with 2, 5, 7, 10, and 20 g/l Sweetzyme Q.

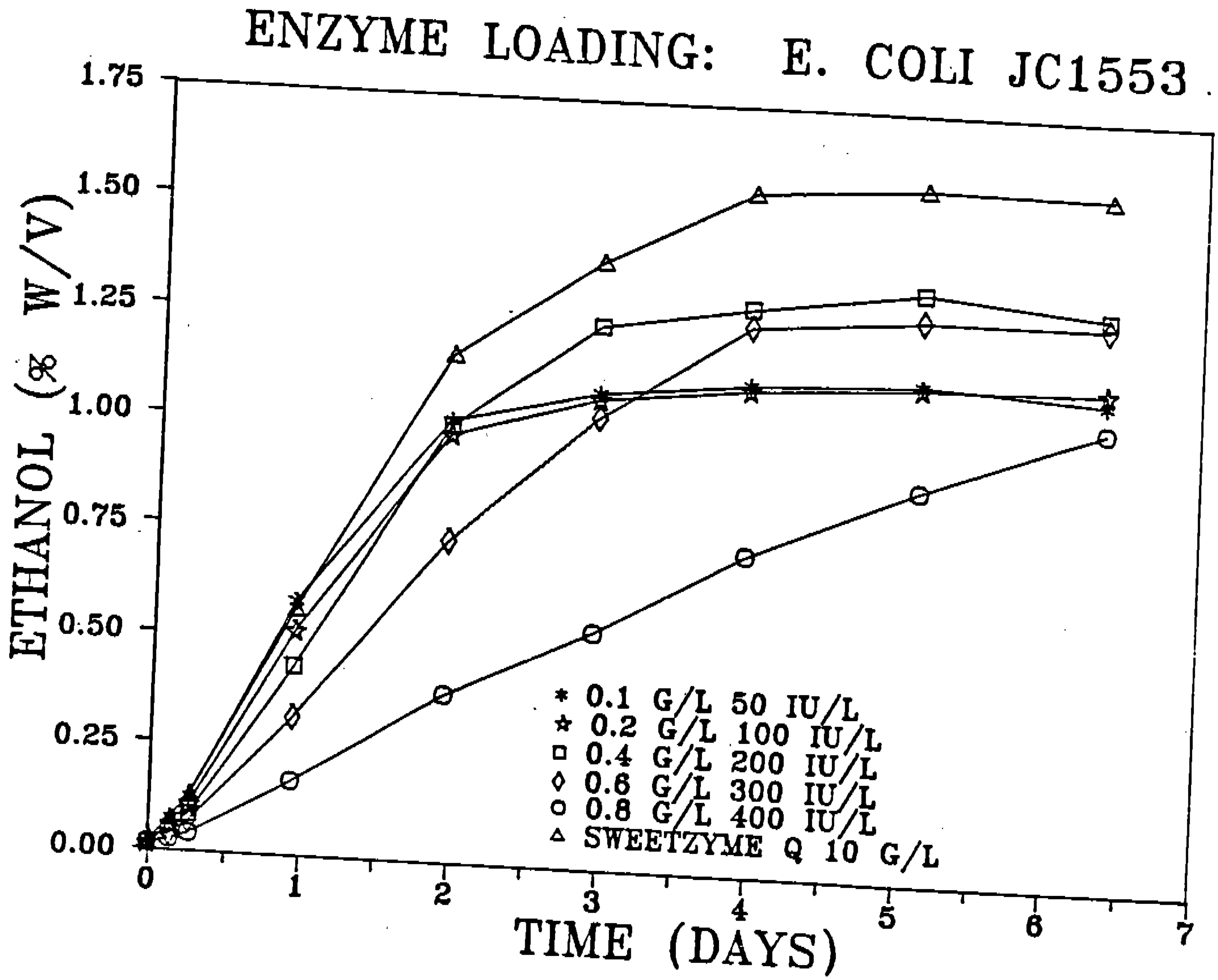

Figure 4. SFIX fermentation of 6% xylose (YEPX) at pH 6.1, 28°C., using recombinant *E. coli* JC1553/pTXI-1-pRK248 cells (0.5 IU/mg dry weight) at 0.1, 0.2, 0.4, 0.6, and 0.8 g/L, with a control fermentation using 10g/L Sweetzyme Q.

required for the metabolism of xylose.

Effect of pH on SFIX

The yield of ethanol in SFIX has been found to increase dramatically as the pH of the fermentation is lowered to below pH 6.0 (Fig. 5). This improvement is probably the result of a number of factors: increased yeast viability, improved ethanol tolerance, lower xylose utilization for cell maintenance and reduced by-product formation. The viability of the yeasts is critical for both high yield of ethanol and for cell recycle.

Immobilization of recombinant E. coli cells

The gene that codes for xylose isomerase in the bacterium *Escherichia coli* has been isolated at SERI and cloned into strains of *E. coli* using plasmid vectors that allow for massive production of the enzyme (Lastick 1986). This procedure, enzyme overproduction, can be used to produce xylose isomerase very inexpensively.

Fermentation at low pH (about pH=5.5) is necessary for the highest ethanol yields but the enzyme loses activity at low pH. It would, therefore, be desirable to improve enzyme activity at lower pH levels. This been achieved by specific immobilization techniques. Recombinant *E. coli* cells, containing overexpressed xylose isomerase, were immobilized by entrapment in a chitosan matrix, providing for significantly higher activity at low pH levels; the immobilized cells were found to have four times the specific activity at pH 5.5 than non-immobilized cells, and an estimated 40 times the activity of non-recombinant, xylose induced *E. coli* (Fig. 6). Lower enzyme requirements and higher yields would be expected when the immobilized enzyme is used in xylose fermentations. In addition to the improving pH compatibility in SFIX, the immobilized cells may be more stable in SFIX and would allow for enzyme recycle.

DISCUSSION

Significant improvements in yield over those previously reported in the literature have been obtained, mainly due to the recognition that high temperature and pH are inimical to the SFIX process. In these studies it was found that reducing the temperature of SFIX from 35°C. to 28°C. resulted in a 77% increase in ethanol yield; decreasing the pH of SFIX from 7.0 to 5.5, at 28°C. doubled the ethanol yield. When SFIX is performed to ferment 6% w/v xylose at a pH of 5.5 at 28°C. an ethanol yield of 60% of the theoretical amount is obtained. This yield is comparable to oxygen mediated fermentation of xylose at this substrate concentration. However, since SFIX is a totally anaerobic process, the necessity of controlling low amounts of oxygen in the oxygen mediated process, which may be difficult and costly in large reactors, is eliminated.

FUTURE THRUSTS

Maintaining the viability of the fermenting yeast to allow for continuous or recycle processes, and improving the activity of the enzyme at lower pH

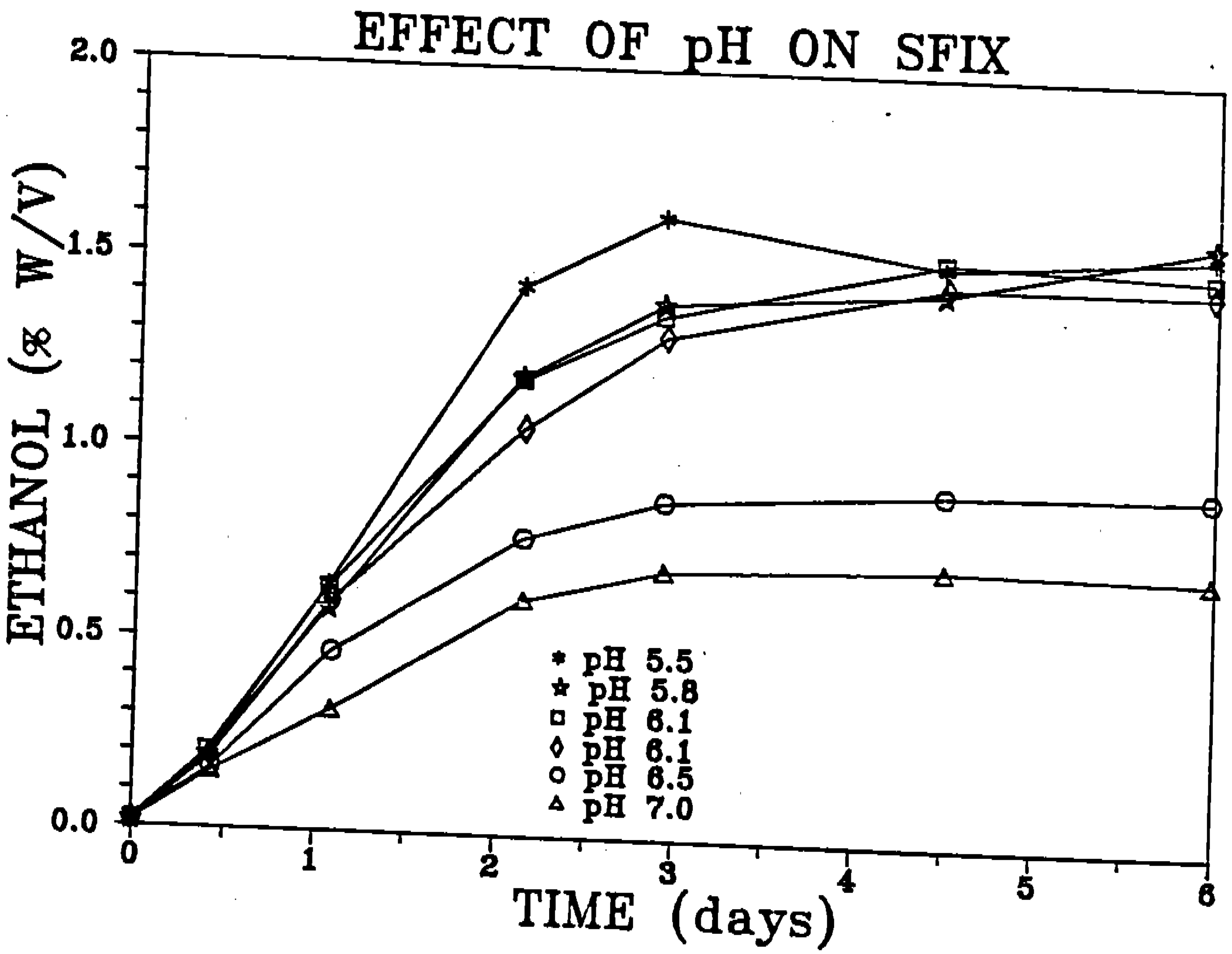

Figure 5. S. pombe Y164 was used to ferment 6% xylose (YEPX) at 28C. using 10 g/L Sweetzyme Q. The pH of the medium was controlled, using 1M Tris base at pH 5.5, 5.8, 6.1, 6.1 (a duplicate control) and 7.0.

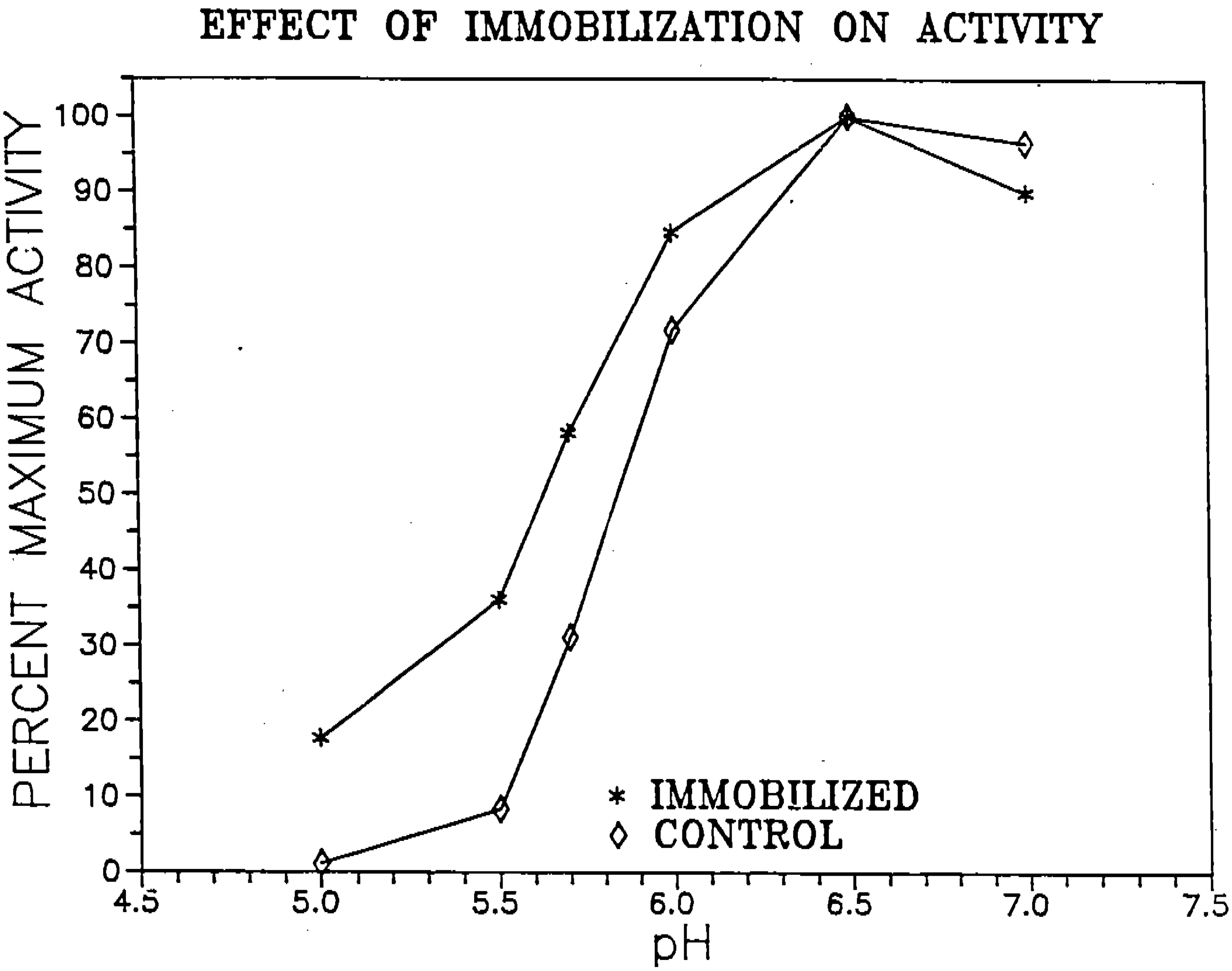

Figure 6. Lyophilized E. *coli* LE392/pTXI-1-pRK248 cells were immobilized by entrapment in chitosan. The immobilized preparation and non-immobilized cells were assayed at pH 5.0, 5.5, 5.7, 6.0, 6.5, and 7.0. The loss in activity by the immobilization procedure was less than 50%.

levels by immobilization techniques are important areas for future research on the SFIX process. Improving xylulose fermenting yeast by subculture, mutagenesis or screening, and the production of immobilized xylose isomerase at very low cost by growing *E. coli* cells to high cell density in fed batch cultures are currently being studied in an attempt to reduce the cost of xylose fermentation and further improve ethanol yields.

REFERENCES

Beck, M. J. 1986. "Factors Affecting Efficiencies of Biomass Fermentation to Ethanol" *Biotech. Bioeng.Symp.* 17: 617-627.

Bruinenberg, P. M., deBot, P.H.M., vanDijken, J. P., and Sheffers, W. A. 1983. "The Role of Redox Balances in the Anaerobic Fermentation of Xylose by Yeasts", *Eur. J. Appl. Microbiol. Biotechnol*. 18: 287-292.

Chiang, L-C., Hsiao, H-Y., Ueng, P. P., Chen, L-F. and Tsao, G. T., 1981a. "Ethanol Production from Xylose by Enzymatic Isomerization and Yeast Fermentation" *Biotech. Bioeng. Symp.* II: 263-274.

Chiang, L-C., Gong, C-S., Chen,L-F., and Tsao, G. T. 1981b. "D-Xylulose Fermentation to Ethanol by *Saccharomyces cerevisiae*," *Appl. Environ. Microbiol.* 42: 284-289.

Chung, I.S., Lee, Y.Y., and Beck, M.J. 1986. "High-Cell Fermentation of Xylose to Ethanol by *Pachysolen tannophilus* Using Continuous Cell Recycle," *Biotech. Bioeng. Symp.* 17: 391-400.

Gong, C-S., Chen, L-F., Flickinger, M. C., Chiang, L-C. and Tsao, G.T. 1981a. "Production of Ethanol from D-Xylose by Using D-Xylose Isomerase and Yeasts", Appl. *Environ.Microbiol.* 41: 430-436.

Gong, C-S, Ladisch, M.R. and Tsao, G.T. 1981b. "Production of Ethanol from Wood Hemicellulose Hydrolysates by a Xylose-Fermenting Yeast Mutant, Candida Sp. XF 217," *Biotech. Lett.* 3: 657-662.

duPreez, V.C., Bosch, M. and Prior, B.A. 1986. "The Fermentation of Hexose and Pentose Sugars by *Candida shehatae and Pichia stipitis*," *Appl. Microbiol. Biotechnol.* 23: 228-233.

Hahn-Hagerdal, B., Berner, S. and Skoog, K. 1986. "Improved Ethanol Production from Xylose with Glucose Isomerase and *Saccharomyces cerevisiae* Using the Respiratory Inhibitor Azide," *Appl. Microbiol. Biotechnol.* 24:287-293.

Hoerecker, B.L. 1974. "D-Xylose and D-Xylulose: Determination with Xylose Isomerase" in *Methods of Enzymatic Analysis*, Academic Press, N.Y. pp 1371-1376.

Hsiao,H-Y, Chiang, L-C., Chen, L-F and Tsao, G.T. 1982. "Effects of Borate on Isomerization and Yeast Fermentation of High Xylulose Solution and Acid Hydrolysate of Hemicellulose", *Enzyme Microb. Technol.* 4:25-31.

Jeffries, T.W. 1981. "Fermentation of Xylulose to Ethanol Using Xylose Isomerase and Yeasts," Biotech. Bioeng. Symp. II: 315-324.

Lastick, S.M., Tucker, M.Y., Mackedonski, V., and Grohmann, K. 1986. "Overproduction of E. coli Xylose Isomerase," Biotech. Lett. 8: 1-6

Maleszka, R. and Schneider, H. 1982. "Fermentation of D-Xylose, Xylitol and D-Xylulose by Yeasts", Can. J. Microbiol. 28: 360-363.

Roman, G.N. Jansen, N.B., and Tsao, G.T. 1984, "Ethanol Inhibition of D-Xylulose Fermentation by Schizosaccharomyces pombe," Biotech. Lett. 6:7-12.

Roman, G.N., Jansen, M.B., Hsiao, H-Y., and Tsao, G.T. 1985. "Kinetic Studies of the Enzymatic Isomerization of Xylose", Enzyme Microb. Technol. 7:129-133.

Schneider, H., Wang, P.Y., Johnson, B.F., and Shopsis, C.. 1981. "Pentose Fermentation by Yeasts," in Current Developments in Yeast Research: Proceedings of the Fifth International Symposium on Yeasts. Pergamon Press, Toronto. pp. 81-85.

Wang, P.Y., Shopsis, C. and Schneider, H. 1980a. "Fermentation of a Pentose by Yeasts," Biochem. Biophys. Res. Comm. 94:248-254.

Wang, P.Y., Johnson, B.F. and Schneider, H. 1980b. "Fermentation of D-Xylose by Yeasts Using Glucose Isomerase in the Medium to Convert D-Xylose to D-Xylulose," Biotech. Lett. 2:273-278.

Vorlop, K-D. and Klein, J. 1981. "Formation of Spherical Chitosan Biocatalysts by Ionotropic Gelation". Biotech Lett. 3:9-14.

OVERPRODUCTION OF XYLOSE ISOMERASE AND CONTROL OF *xylA* COPY NUMBER IN LARGE-SCALE FERMENTATIONS OF *ESCHERICHIA COLI*

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ABSTRACT

Cells of *Escherichia coli* have been transformed with the plasmids pTXI-1 and pRK248. Plasmid pTXI-1 contains a copy of the gene which encodes xylose isomerase (*xylA*) and the strong promoter P_L from bacteriophage Lambda. Plasmid pRK248 contains cI857 from Lambda which encodes the temperature-sensitive repressor of promoter function. Upon temperature upshift from 32°C to 42°C cultures of *E. coli* (pTXI-1/pRK248) are induced for massive overproduction of xylose isomerase. The enzyme converts non-fermentable xylose to xylulose - a sugar which can be fermented readily to ethanol. The objectives of this study are to resolve the major problems associated with overproduction of xylose isomerase in *E. coli*. These include, at the molecular level, the reversion and recombination of cI857 and fluctuation in plasmid copy number, the variation in enzyme stability (biochemical level) and, at the physiological level, the need to maintain growth of cultures at high-cell density.